WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 10, 1395-1405. Research Article ISSN 2277– 7105

"EMBEDDING POTENTIAL BIOINOCULANT STRAIN FOR EFFECTIVE SOIL CONDITIONER PRODUCTION"

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Article Received on
3 Aug 2015,

Revised on 23 Aug 2015,
Accepted on 12 Sep 2015

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ABSTRACT

Biofertilizer is the resource and producer of plant nutrients and promotes growth by increasing supply of essential nutrients and growth induced to the target crop when applied to plant surfaces, seed and soil. Biofertilizers are environmental friendly fertilizers that aid in Nitrogen fixation, Phosphate solubilization and Potassium mobilization. But studies regarding organisms solubilizing secondary nutrients like sulphur and magnesium are yet to be carried out optimally. Sulphur and Magnesium are secondary nutrients required in small quantities. In the present investigation Sulphur oxidizing bacteria(SOB) and Magnesium oxidizing bacteria (MOB) were isolated from Sugarcane(Ladewadgaon, Dist.Beed, Maharashtra) & Citrus rhizosphere (Rajiv Gandhi Biotechnology Centre, RTMNU, Nagpur). The isolates were characterized by using Morphological and biochemical, cultural tests and screened by PCR using specific oligonucliotide primers. Isolates were sequenced by 16S r RNA sequencing for molecular characterization and identification. *Bacillus sp. & Serratia marcescens sp. were* found to be competent isolates for sulphur & magnesium oxidation respectively. Further green house studies were conducted to see their impact of plant growth.

KEYWORDS: Biofertilizers, Sulphur, Magnesium, Secondary, PCR, 16s r RNA.

INTRODUCTION

Biofertilizer is one of the approaches to increase the production in sustainable agriculture. Soil conditioner is the resource and producer of plant nutrients and promotes growth by increasing supply of essential nutrients and growth induced to the target crop when applied to

plant surfaces, seed and soil. The plant is greatly influenced by a wide range of these nutrients. Soil is the reservoir of nutrients required by plants. But it cannot sustain the demand by crops continuously, season after season and hence farmers have resorted to application of fertilizers. Even here farmers apply major nutrients like Nitrogen, Phosphorus and Potassium often and seldom the secondary and micronutrients. Magnesium and Sulphur are secondary nutrients required in substantial quantities by crops but applied rarely.[1] Sulphur is considered as the fourth major plant nutrient after N, P and K, and is one of the sixteen nutrient elements which are essential for the growth and development of plants. Sulphur is one of the essential plant nutrients and it contributes to yield and quality of crops. Sulphur oxidizers are involved in oxidation of elemental sulphur to plant available sulphate. Sulphur is the key element for higher pulse production and plays an important role in the formation of proteins, vitamins and enzymes.[2] *Thiobacilli sp.* play a significant role in sulphur oxidation in soil. Sulphur oxidation is the mainly important step of sulphur cycle, which improves soil productiveness. Subsequently, the sulphide can be oxidized by sulphur oxidising bacteria to produce sulphate.[3] Next secondary nutrient Magnesium, plays an essential role in stepping up the growth and quantitative as well as qualitative features of the plant. In reclaimed sandy soils, foliar applications of macro and micro nutrients are widely used and preferable and show the way to significant increase in growth and productivity of some crops.[4] Magnesium is absorbed as the Mg2+ ion and is mobile in plants, moving from the older to the younger leaves. To correct Magnesium deficiency in soil, use Dolomitic lime when lime is needed; use soluble sources of Magnesium when lime is not needed. Magnesium supplementation may be needed for crop production.[5]

Magnesium and Sulphur are secondary nutrients required in sustainable quantities by crops but applied rarely. These secondary nutrients are taken up in smaller quantities but are essential for plant growth.[6] In this study we have isolated bacteria that have ability to oxidize sulphur & magnesium from the rhizophere of sugarcane and orange. Further Biofertilizers were made with the potential isolates and green house studies were conducted to validate its growth enhancement property.

MATERIALS AND METHODS

The studies were conducted at Rajiv Gandhi Biotechnology Centre RTM Nagpur University, Nagpur during the year 2014-15.

Collection of soil samples

Isolation was performed using soil samples collected from two field sites viz., Citrus rhizosphere soil Orange (*Citrus sinensis)* Field, RGBC, RTM Nagpur University, Nagpur, Maharashtra) & Sugarcane *(Saccharum officinarum L.)* rhizosphere soil Ladewadgaon, Dist.Beed, Maharashtra.

Isolation of sulphur oxidizing bacteria

Isolation was performed by using samples collected from rhizosphere soil sample of Sugarcane field from (Ladewadgaon, Dist.Beed, Maharashtra).

The media used for the isolation of sulphur oxidizing bacteria was thiosulphate broth having 5.0 g $Na_2S_2O_3$, 0.1 g K_2HPO_4, 0.2 g $NaHCO_3$, 0.1 g NH_4Cl in 1000 ml distilled water, with pH 8.0. 0.0025 g of Bromo phenol blue (BPB) was used as an indicator.[7] The obtained isolates were inoculated in the growth media incubated at 32 ^oC for 15 days. The isolates were screened for their efficacy to reduce the pH 8.0.

For qualitative screening, the isolated bacteria from agar plate were further grown on the thiosulphate agar and broth.[8] The selected isolates were further studied for their morphology, Gram reaction, colony characters etc.

Isolation of Magnesium Solubilizing Bacteria

Isolation was performed by using samples collected from Sweet orange field rhizosphere soil (Sweet orange, RTM Nagpur University, Nagpur).

The solubilization of Magnesium was investigated in liquid culture and in soil incubation studies. Basal medium (Glucose-10g; $(NH_4)SO_4$ 1.0g; KCl-0.2g; K_2HPO_4 -0.1g; $MgSO_4$ -0.2g; DIS-1000ml; pH 7.0) was prepared and dispensed in 100ml quantities in 500ml Erlenmeyer flasks to which talc, dolomite and Magnesium trisilicate were added separately at 0.25% levels.[1] The obtained isolates were inoculated in the growth media incubated at 32 ^oC for 15 days. The isolates were screened for their efficacy to reduce the pH 7.0. The selected isolates were further studied for their morphology, Gram reaction, colony characters etc.

Morphological Biochemical and Cultural Characterization

The selected isolates were plated in Sodium Thiosulphate and Magnesium Trisilicate agar medium to study colony morphology. The isolates were subjected to Gram staining and

Motility test. Also biochemical tests like Indole Acetic acid test and IMViC Test were performed and the results were recorded.

Isolation of Genomic DNA From Bacteria

The genomic DNA extraction and PCR techniques are found to be the very fast and precise methods for evaluating Sulphur oxidizing and Magnesium oxidizing bacterial isolates ability. Total genomic DNA was isolated from SOB and MOB isolates. Genomic DNA isolation was carried out from the isolates by using the phenol/chloroform/ isoamyl alcohol method.[9] The total genomic DNA isolated from SOB and MOB isolates was amplified by PCR. Polymerase Chain Reaction was performed using the Eppendorf Master Cycler, Gradient (Eppendorf, Germany**).** PCR amplification was carried out using 16S rRNA universal primer.[10] Screened isolates were further characterised by amplification with oligonucleotides two 16S F & 16S R primers.

Preparation of Biofertilizer

Bioinoculant are used to prepare as carrier based inoculants containing effective microorganisms. Among various type of Bioinoculant which includes Sulphur oxidizing bacteria (SOB) and Magnesium oxidizing bacteria (MOB). Basically, the carrier based inoculant of these bacteria can be prepared by a standard procedure, which is routinely being followed for the said preparations.[11]

Green House Study

Soil was collected from agricultural field, sterilized at 121^0C for 15 min at 15 lbs pressure. The sterile soil was cooled to room temperature and dispensed in to sterile pots and watered. The soil sample with concentration of 25% co- inoculums of Sulphur oxidizing, Silicate Solubilizing & Magnesium oxidizing bacteria (with charcoal) were showed in pots and were maintained in green house.

RESULT AND DISCUSSION

Growth in Selective Media

Isolation of Sulphur oxidizing Bacteria

Qualitative screening of isolated Sulphur Solubilizing Bacteria (SOB).

The Thiosulphate medium for isolating Sulphur Oxidising Bacteria (SOB) was a simple way to detect SOB through the formation on agar plates containing Sodium Thiosulphate as a only

'Sulphur' source. The isolates were facultative autotrophic in nature. Isolation of Autotrophic sulphur oxidizers from different ecological niches. The sulphur oxidising bacterial isolates obtained from the Chick pea soil could reduce the pH up to 4.3 to 4.2 from the initial pH 8.0 of thiosulphate broth within 11 days of incubation. The isolates were selected based on the better pH reduction ability on the Bromophenol blue containing sulphur oxidising broth and agar medium by changing the colour of the media purple to colourless (Fig.1). The Sulphur oxidising bacterial isolates obtained from the Sugarcane soil initial pH 8.0 of thiosulphate broth within 11 days of incubation. *In vitro* studies showed that this *Bacillus* can solubilize Sulphur from the minerals. It is imperative that Sulphur oxidizing bacteria can liberate sulphate from bearing S minerals. *Thiobacillus thiooxidans* cells oxidized elemental S to SO_4^{2-} in growth medium.[12]

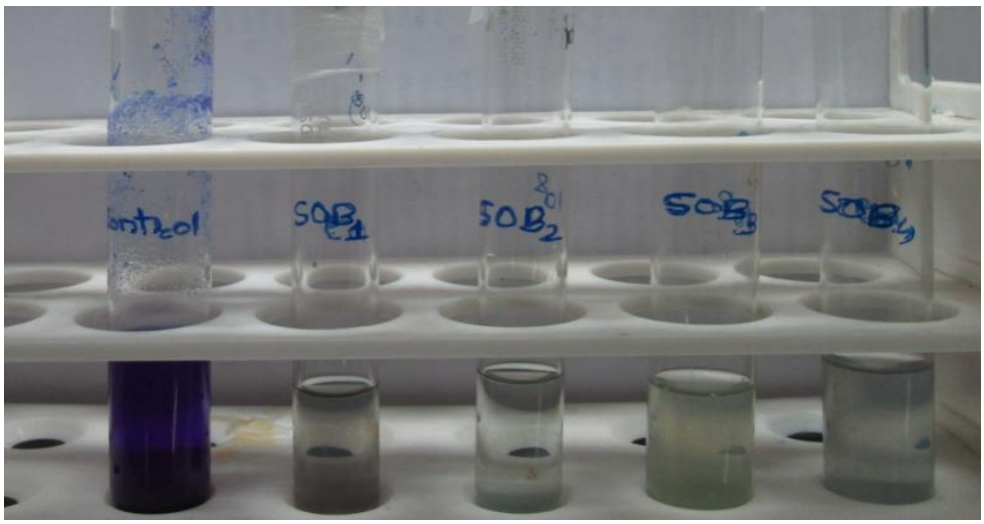

Figure 1: Screening of sulphur oxidising bacteria on thiosulphate broth supplied with bromophenol blue as an indicator.

Isolation of Magnesium Oxidising Bacteria

Qualitative screening of isolated Magnesium Oxidising Bacteria (MOB)

Use of Magnesium Trisilicate for isolating Magnesium Oxidising Bacteria (MOB) was a simple way to detect MOB through the formation on agar plates containing Magnesium Trisilicate as a sole "Magnesium" source Fig (2). *In vitro* studies showed that this *Serratia marcescens sp.* can solubilize Magnesium from the Magnesium minerals. In the current study utilization of Magnesium by the *Serratia marcescens sp.* under *in vitro* condition was clearly recognized. The capability to solubilise Talc, Dolomite and Magnesium trisilicate *in vitro* and when added to the soil point to that this bacterium can attack other silicate mineral in soil and liberate Magnesium.[13]

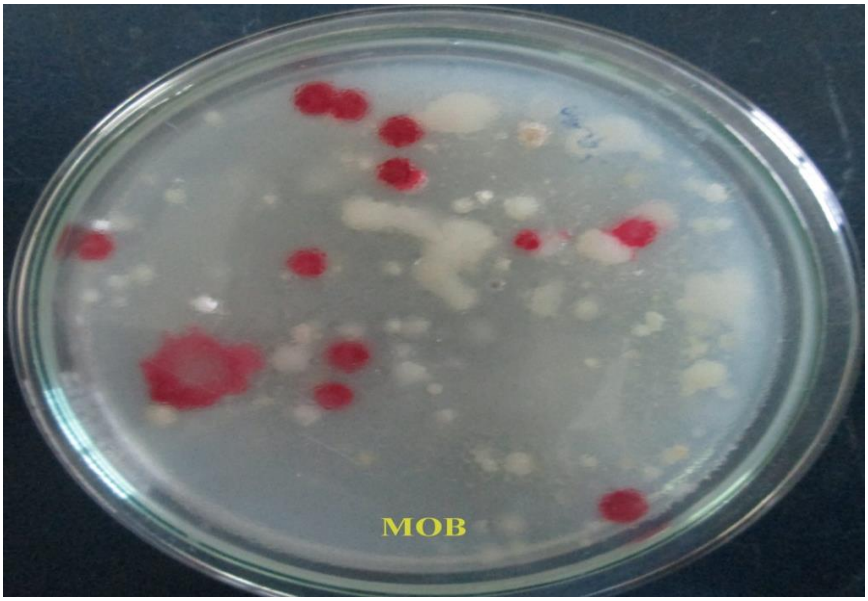

Figure 2: Isolation of Magnesium oxidizing bacteria.

Figure 3: (A-B) Inoculation of Sulphur Oxidising Bacteria (SOB) and Magnesium oxidizing bacteria (MOB).

Morphological and Biochemical characterization of isolates

Three Bacterial isolates were characterized on the basis of morphological and biochemical tests. The result obtained from biochemical tests like Indole Acetic acid test and IMViC Test are depicted in Table 1.

Table 1: Showing Morphological and Biochemical characterization of bacterial isolates.

S.No	Test	SOB	MOB
1	Gram reaction	Positive	Negative
2	Colony morphology	Rods	Coccobacilli
4	Indole Acetic Acid Test	Negative	Negative
5	Indole test	Negative	Positive
6	Methyl red test	Positive	Positive
7	Vogesproskauer test	Positive	Negative
8	Citrate Test	Positive	Negative

16s RNA SEQUENCING CHARECTERIZATION

Sulphur Oxidizing Bacteria (SOB) of 16s RNA Sequencing Characterization

16s rRNA and gene amplification of SOB isolates

The selective SOB isolates were subjected to PCR using specific primers like 16S rRNA oligonucleotide primers. When the genomic DNA of selected SOB isolates were amplified by PCR using primers, a major amplification band of 1500bp was observed and 16sr RNA yielded 1623 bp band which was notified by running the PCR product on agarose gel.

The sequence information for nucleotide was obtained by comparison of the sequence and it showed highest similarity (90 % similarity) with the sequence of *Bacillus sp.* Respectively (Fig. 4 & 5) & table 2.

Table 2: Identification of bacteria.

Sl No	Sample ID	Identification (BLAST)	Percentage similarity
1	SOB	*Bacillus sp*	90

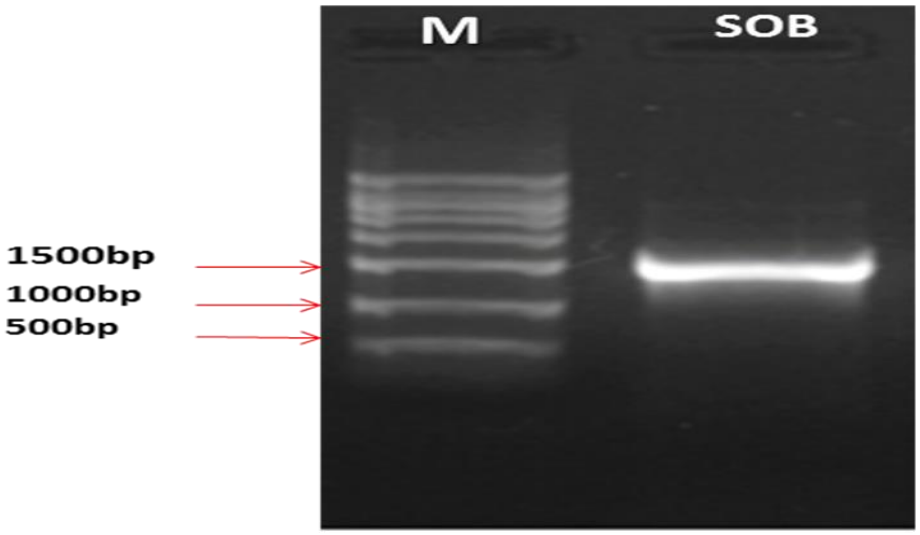

Figure 4: Gel electrophoresis of amplified of SOB isolates through PCR.

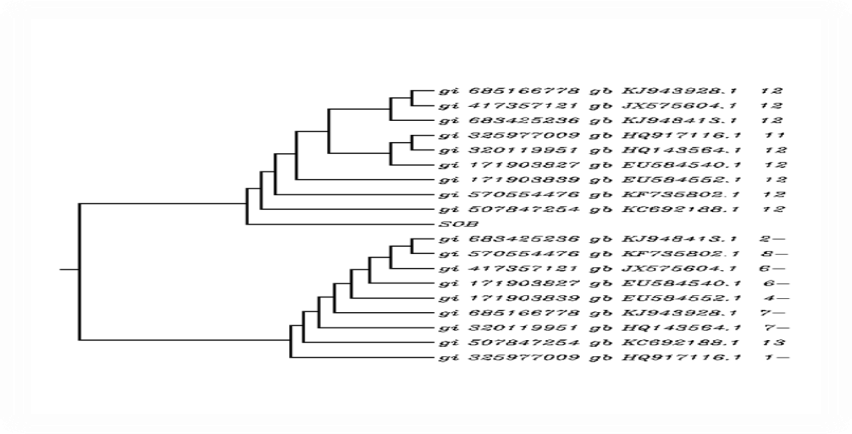

Figure 5: Phylogenetic analysis of obtained sequence of 16S Region with *Bacillus sp.* sequences from Genbank.

Magnesium Oxidising Bacteria of 16s RNA Sequencing Characterization

16s rRNA and gene amplification of MOB isolates

The selective MOB isolates were subjected to PCR using specific primers like 16S rRNA oligonucleotide primers. When the genomic DNA of selected MOB isolates were amplified by PCR using primers, a major amplification band of 1500bp was observed and 16sr RNA yielded 1477 bp band which was notified by running the PCR product on agarose gel.

The sequence information for nucleotide was obtained by comparison of the sequence and it showed highest similarity (97% similarity) with the sequence of *Serratia marcescens* respectively (Fig.6 & 7) & table 2.

Table 3: Identification of bacteria.

Sl No	Sample ID	Identification (BLAST)	Percentage similarity
1	MOB	*Serratia marcescens*	97

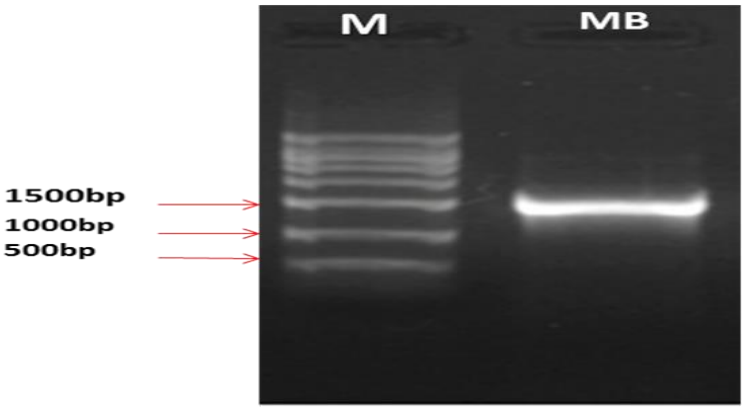

Figure 6: Gel electrophoresis of amplified of MOB isolates through PCR.

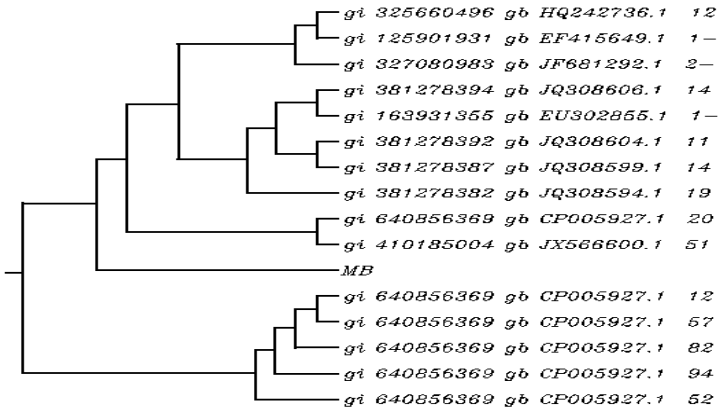

Figure 7: Phylogenetic analysis of obtained sequence of 16S Region with *Serratia sp.* sequences from Genbank.

GREEN HOUSE STUDY

The results of present Green house study clearly showed that selected isolates effectively enhance the plant growth, total Sulphur and Magnesium uptake and soil fertility.

The result of present study green house visibly showed growth enhancement ultimately and development of plant under green house condition. The biofertilizer use 25% and 75% soil. Field trials were conducted under green house condition to study the influence of concentration of Sulphur Oxidizing and Magnesium Oxidizing co- inoculums. Pots were placed in a greenhouse under a 25/18ºC day/night temperature regime with a 14-h photoperiod. Showing shoot length Sulphur & Magnesium soil conditioner treatment recorded maximum length of shoot and found significantly superior over rest of control treatment (Figs.8 A&B).

Figure 8: Growth promotion of plant with Sulphur Oxidizing (A) & Magnesium Oxidizing Bacterial (B) soil conditioner.

CONCLUSION

In current study we were able to isolate Sulphur & Magnesium oxidizing bacteria, it was found that these can substitute the chemical fertilizer, might be used to reduce the alkalinity of soil by neutralization phenomenon through organic acid exudation and can survive in the soil system to retain the Sulphur and Magnesium potential for long time. Use of these SOB as bio-inoculants can be incorporated to enhance sulphur oxidation in soil and to increase soil available sulphate to minimize the S-fertilizer application. The present study revealed that the isolates SOB & MOB have the potential for developing into a farmer friendly bioinoculant or soil conditioner for enhancing the Sulphur & Magnesium nutrition, growth promotion in several crops after testing under field conditions.

ACKNOWLEDGMENT

The authors are grateful to DBT, Govt. of India for providing financial support to carry out this project dissertation work.

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